Null Results in Brief

Polymorphisms in Interleukin -2, -6, and -10 Are Not Associated with Gastric Cardia or Esophageal Cancer in a High-Risk Chinese Population

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Introduction

Chronic inflammation is thought to contribute to the development of cancer (1). Progressive inflammation leads to activation of inflammatory cytokines, recruitment of inflammatory cells, generation of free radical species, and subsequent malignant transformation. Individual responses to inflammation, mediated by genetic variation, may influence the degree of inflammation and thus the risk for cancer.

Interleukin-2 (*IL-2*) is a Th1 cytokine with well-characterized single nucleotide polymorphisms (SNP) in the promoter (−384, rs2069762) and exon 1 (+114, rs2069763; ref. 2). Interleukin-6 (*IL-6*) is a key mediator of the Th2 response that has a promoter SNP (−174G/C, rs1800795) associated with increased *IL-6* production (3). Increased levels of *IL-6* were associated with worse prognosis in advanced gastric cancer (4). Interleukin-10 (*IL-10*) is an immunoregulatory Th2 cytokine with a polymorphic promoter (−1082, rs1800896; −819, rs1800871; and −592, rs1800896), with variants correlated with increased *IL-10* production (5). These *IL-10* polymorphisms have been associated with nongardia gastric cancer risk (6).

The population of Linxian, a county in north-central China, is at very high risk for esophageal squamous cell carcinoma (ESCC) and gastric cardia adenocarcinoma (GCC), with age standardized incidence rates for the two cancers >125/100,000 per year (7, 8). The cause of these extraordinary rates is most likely multifactorial. Higher risks for ESCC and GCC have been associated with age, family history (9), low levels of antioxidants (10-12), tooth loss (13), and polymorphisms in methylenetetrahydrofolate reductase (8) in this population. Tobacco and alcohol use, leading risk factors for ESCC in Western countries, have only a minor role in this population (14).

We hypothesized that genetic variations within key molecules of the Th1-Th2 pathways (*IL-2*, *IL-6*, and *IL-10*) underlie differential responses to inflammation and may be associated with the risk of developing GCC and/or ESCC in this high-risk Chinese population.

Materials and Methods

Trial Description. Between 1986 and 1991, the National Cancer Institute and the Cancer Institute of the Chinese Academy of Medical Sciences completed the Nutrition Intervention Trials in Linxian (8, 15). A stratified case-cohort design was used to select individuals for inclusion from the cohort of all eligible participants in the general population and dysplasia trials. Among 4,005 trial participants with sufficient genomic DNA, all eligible incident cases of ESCC (n = 130) or GCC (n = 91) that occurred between May 1991 and April 1996 were included in the study. A random sample was selected and stratified on and matched to the age and sex distribution of the combined cancer cases of all eligible trial participants to serve as the subcohort (control, n = 454) group. All participants were monitored in an active surveillance program, which allowed near complete case ascertainment with little or no loss to follow-up.

All cases of esophageal cancer were squamous cell carcinomas and all gastric cancer cases were GCCs (in the proximal 3 cm of the stomach), which are ~3 times more common than body of stomach cancers in Linxian. Genomic DNA was purified from blood using standard methods.

Genotype Analysis. Investigators blinded to all patient identifiers and information did the genotype analysis. DNA plates were arranged such that 20% of the samples were duplicated. Genomic DNA was amplified by PCR with MJ Research model PTC-225 thermal cyclers (MJ Research, Waltham, MA) under the following conditions: 5 ng genomic DNA, 0.2 μmol/L of each primer, 200 μmol/L of each deoxynucleotide triphosphate, 2 mmol/L $MgCl_2$, 0.5 unit AmpliTaq Gold DNA polymerase (ABI Perkin-Elmer, Foster City, CA), and the manufacturer's

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buffer with the following primer pairs; *IL-2*: CCATTCTGAAACAGGAAACC and ATGTACAGGATGCAACTCCT, *IL-6*: TTGTCAAGACATGCCAAGTGC and GGAGATGTCTCTGAGGCTCATTCTG, and *IL-10*: ATCCAAGACAACACTACTAA and TAAATATCCTCAAAGTTCC. Annealing temperatures were 59°C, 62°C, and 45°C, respectively. PCR products (1.0 μL each) were pooled and incubated for 60 minutes at 37°C with 1 unit shrimp alkaline phosphatase and 1 unit exonuclease I. Enzymes were inactivated by incubation at 75°C for 15 minutes.

Single base extension using gene-specific primers was done according to the manufacturer's protocol (ABI Prism SNaPshot Multiplex System from Applied Biosystems, Foster City, CA) with the following modifications: 1 μL reaction mix, 5 μL pooled purified PCR product, single base extension primers, and water to a final reaction volume of 10 μL. Single base extension primers were (lower case letters indicate nonannealing bases) *IL-2* −384T/G (rs2069762) ctgactgactATATGCTATTCACATGTTCAGTGTAGTTTTA (0.5 μmol/L), *IL-2* +114G/T (rs2069763) ctgactgactACACAGCTACAACTGGAGCATTTACT (0.5 μmol/L), *IL-6* −174G/C (rs1800795) gactgactgactgactgactgactTTTCCCCCTAGTTGTGTCTTGC (0.17 μmol/L), *IL-10* −1082G/A (rs1800896) ACTACTAAGGCTTCTTTGGGA (0.83 μmol/L), *IL-10* −819C/T (rs1800871) GGTGTACCCTTGTACAGGTGATGTAA (0.83 μmol/L), and *IL-10* −592C/A (rs1800872) AGCCTGGAACACATCCTGTGACCCCGCCTGT (0.83 μmol/L). Reactions consisted of 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds and 37°C for 60 minutes with 5 units shrimp alkaline phosphatase followed by 15 minutes at 75°C. Purified reaction (0.5 μL each) was run on an ABI Prism 3100 Genetic Analyzer according to the manufacturer's instructions. Analysis was done using ABI Prism GeneScan version 3.7 and Genotyper version 3.7 (Applied Biosystems).

Statistical Analysis. Hardy-Weinberg equilibrium was determined in the subcohort. All *P*s reported are two sided. Relative risks and 95% confidence intervals were estimated using the case-cohort estimator for the Cox proportional hazards models (Epicure, Hirosoft International Corp., Seattle, WA). Risk estimates were determined using subjects homozygous for the most prevalent genotype as the reference group. All estimates came from models stratified on the six sex-age sampling strata. In addition to the matching factors, additional stratum-specific age terms for continuous age were used to adjust for variation within age strata and variables for tobacco smoking, alcohol drinking, and trial. Nested models were compared using score tests. We tested the proportional hazards assumption for each main effect (Genotype) using a time-dependent covariate (Genotype × Follow-up time). This test was nonsignificant ($P > 0.05$) in all cases.

Results

Frequencies of the *IL-2* and *IL-10* SNPs in the subcohort, GCC, and ESCC individuals were analyzed and are shown in Table 1. These data show no association

Table 1. Frequency, RR, and 95% CI for *IL-2* and *IL-10* SNPs

Genotype	Subcohort* [*n* (%)]	GCC				ESCC			
		n (%)	Relative risk†	95% Confidence interval	$P_{2\ df}$‡	*n* (%)	Relative risk†	95% Confidence interval	$P_{2\ df}$‡
IL-2 −384									
G/G	96 (25.3)	16 (19.3)	1.00	Reference	0.08	35 (31.5)	1.00	Reference	0.41
G/T	174 (45.9)	47 (56.6)	1.91	(1.01–3.63)		43 (38.7)	0.69	(0.40–1.19)	
T/T	109 (28.8)	20 (24.1)	1.21	(0.58–2.51)		33 (29.7)	0.83	(0.46–1.48)	
IL-2 +114									
G/G	149 (39.5)	33 (40.2)	1.00	Reference	0.49	44 (39.6)	1.00	Reference	0.93
G/T	148 (39.3)	35 (42.7)	1.18	(0.69–2.01)		41 (36.9)	1.05	(0.64–1.73)	
T/T	80 (21.2)	14 (17.1)	0.77	(0.38–1.55)		26 (23.4)	1.12	(0.62–2.01)	
IL-10 −1082									
G/G	284 (73.8)	60 (71.4)	1.00	Reference	0.57	81 (70.4)	1.00	Reference	0.44
G/A	81 (21.0)	20 (23.8)	1.28	(0.72–2.27)		26 (22.6)	1.32	(0.78–2.25)	
A/A	20 (5.2)	4 (4.8)	0.74	(0.24–2.26)		8 (7.0)	1.48	(0.59–3.72)	
IL-10 −819									
T/T	170 (44.5)	37 (44.1)	1.00	Reference	0.64	53 (45.7)	1.00	Reference	0.72
T/C	163 (42.7)	38 (45.2)	0.92	(0.55–1.54)		46 (40.0)	0.83	(0.52–1.34)	
C/C	49 (12.8)	9 (10.7)	0.68	(0.30–1.51)		17 (15.0)	1.00	(0.52–1.94)	
IL-10 −592									
C/C	171 (44.3)	36 (42.9)	1.00	Reference	0.70	51 (42.9)	1.00	Reference	0.95
C/A	166 (43.0)	39 (46.4)	0.95	(0.57–1.59)		51 (42.9)	0.96	(0.60–1.54)	
A/A	49 (12.7)	9 (10.7)	0.71	(0.31–1.58)		17 (14.3)	1.07	(0.55–2.08)	

*The subcohort was tested by χ^2 to determine if the population meets the assumption of Hardy-Weinberg equilibrium for each: *IL-2* −384, $P = 0.12$; *IL-2* +114, $P < 0.01$; *IL-10* −1082, $P \leq 0.01$; *IL-10* −819, $P = 0.32$; *IL-10* −592, $P = 0.38$.

†Relative risks and 95% confidence intervals were generated from models stratified on age and sex with additional adjustment from continuous age variables for each stratum and variables for smoking, drinking, and trial.

‡This *P* comes from the score test for the addition of all the genotype indicator variables to the base model simultaneously. It assesses the overall association between genotype and cancer.

between polymorphisms at the SNPs studied in these genes and risk for GCC or ESCC. The *IL-6* −174G/C SNP had an allele frequency of <1% in the subcohort, GCC, and ESCC cases. Therefore, risk estimates for *IL-6* −174G/C were not calculated.

Two of the SNPs studied, *IL-2* +114 and *IL-10* −1082, were not in Hardy-Weinberg equilibrium in cases or in the subcohort of this population. This precluded haplotype analysis of the two *IL-2* SNPs. Haplotypes constructed using Phase software version 1 (16) of the *IL-10* −819 and *IL-10* −592 SNPs did not show an association between specific haplotypes and risk of GCC or ESCC.

Discussion

The host response to chronic inflammation most likely plays a critical role in the pathogenesis of malignant transformation and genetic variation in molecules important in the inflammatory response may be associated with risk for the development of cancer at several sites, including the gastrointestinal tract. Our power to detect a relative risk of ≥2 ranged from 0.76 to 0.91 for ESCC and from 0.63 to 0.81 for GCC. In a separate study of the interleukin-8 chemokine pathway, we showed that variants in the interleukin-8 gene were associated with increased risk of GCC in this population (17). Because interleukin-8 is a chemoattractant for neutrophils and has little effect on the balance between Th1 and Th2, we conducted separate analyses.

We chose to study *IL-2*, *IL-6*, and *IL-10* because they represent three different mechanisms of the cytokine immune response. *IL-2*, a well-characterized Th1 cytokine, was not associated with risk of GCC or ESCC in our population. Others have shown that increased levels of *IL-6* were associated with worse prognosis in advanced gastric cancer (4) but variation in *IL-6* was not shown to be associated with gastric cancer risk (6). In our population, the *IL-6* −174G/C variant was rare, illustrating the importance of determining SNP frequency within the population of interest. Variation in *IL-10*, an anti-inflammatory cytokine, is associated with noncardia gastric cancer (6). There is no association between *IL-10* variants and GCC in this population. There may be different mechanisms underlying the genetics of cardia versus noncardia gastric cancer.

We were unable to assess the risk based on haplotypes formed by SNPs in *IL-2* and *IL-10* because *IL-2* +114G/T and *IL-10* −1082G/A were not in Hardy-Weinberg equilibrium in this population. We believe that genotype error is unlikely because questionable base calls identified by Genotyper software were read manually; questionable results were verified and concordance was observed between duplicate samples. Immunologic SNPs are often under a degree of selective pressure based on the population studied, which is probably a contributing factor to the results seen in this study.

Overall, this study showed that variations at the sites studied in *IL-2*, *IL-6*, or *IL-10* were not associated with GCC or ESCC risk. Other genes important in the inflammatory response should be explored as they could contribute to the development of ESCC or GCC in this or other populations.

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